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Spontaneous, Persistent Infection of a B-Cell Lymphoma With Adenovirus

Phyllis Flomenberg, Victoria Piaskowski, Joseph Harb, Annette Segura, and James T. Casper

Departments of Medicine, Pathology, and Pediatrics, Medical College of Wisconsin, John L. Doyne Hospital, and Children's Hospital of Wisconsin, Milwaukee, Wisconsin

An adenovirus culture-positive lymphoblastoid cell line was derived from a bone marrow transplant recipient with fatal B-cell lymphoproliferative disease and adenovirus pneumonia. At autopsy, focal areas of the lymphoma infiltrating the patient's lung were positive for adenovirus proteins by immunohistochemical staining. The Epstein-Barr virus-transformed B-cell line Mk, established from pleural fluid cells, contained adenovirus virions in both the nucleus and the cytoplasm by electron microscopy. The majority of Mk cells expressed adenovirus proteins and produced a high level of infectious adenovirus by plaque assay analysis. However, in contrast to the rapid cell death induced by adenovirus in other permissive cell lines, Mk was maintained stably in tissue culture for 6 months. These data indicate that adenoviral replication is not sufficient for cell lysis and confirm that adenovirus can cause persistent infection in human lymphoid cells in vivo.

KEY WORDS: electron microscopy, immunohistochemical staining, viral plaque assay

INTRODUCTION

Adenovirus is becoming an increasingly important viral pathogen in immunocompromised hosts, in whom infection has been associated with a wide spectrum of diseases, including colitis, cystitis, pneumonia, hepatitis, and encephalitis [Horwitz, 1990; Hierholzer, 1992]. No therapy has been documented to be of benefit against adenovirus, and invasive disease is usually fatal. We recently described a high incidence of adenovirus infection (21%) and disease (7%) in T-cell depleted bone marrow transplant recipients [Flomenberg et al., 1994]. The source of the adenovirus infections in these patients, however, is not clear. Based on evidence that adenovirus can persist in nonimmunocompromised hosts, e.g., adenoviruses can be shed in the feces for months to years after acute infection [Fox et al., 1977], reactivation of persistent infection may play a role. Limited serologic data in bone marrow and solid organ transplant recipients in whom adenovirus disease developed supports this hypothesis [Purtilo et al., 1985; Koneru et al., 1990].

The sites of persistent infection from which adenovirus may reactivate in immunocompromised patients have not been well defined. Lymphocytes have been postulated to play a role because adenovirus has been isolated from the adenoids and tonsils of asymptomatic individuals [Van der Veen and Lambriex, 1973]. In addition, adenovirus DNA was documented in peripheral blood mononuclear cells (PBMC) by Southern blot analysis from a majority of healthy individuals tested by Horvath et al. [1986]. However, in these two studies, the specific infected cell type was not identified. In vitro, adenovirus infection in PBMC or lymphocyte cell lines has been difficult to study because it is typically inefficient and does not cause cytopathic effect (CPE). Experimental adenovirus infection of lymphocyte and monocyte cell lines, however, has resulted occasionally in a low level persistent infection [Andiman and Miller, 1982; Silver and Anderson, 1988; Chu et al., 1992].

In this study, a de novo, chronic, productive adenovirus infection was documented in a lymphoblastoid cell line derived from a bone marrow transplant recipient who developed both adenovirus type 2 pneumonia and B-cell lymphoproliferative disease (BLPD). The cell line was evaluated by cell surface immunophenotyping, electron microscopy (EM), chromosome analysis, and examination for Epstein-Barr virus (EBV) and adenovirus antigens. The adenovirus isolated from the lymphoblastoid cell line was tested for the ability to infect and cause CPE in other cell types in comparison to the Ad2 prototype. In addition, viral plaque assay and immunohistochemical analyses were utilized to determine the titer of adenovirus produced and the uniformity of the infection within the cell line.

MATERIALS AND METHODS

Cell Lines and Viruses

The Mk cell line was established from a pleural fluid specimen obtained from the patient described below.

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Address reprint requests to Dr. Phyllis Flomenberg, Thomas Jefferson University, 329 Jefferson Alumni Hall, 1020 Locust St., Philadelphia, PA 19107.

Pleural fluid cells (1.5×10^6 cells/ml) consisting of >85% lymphoblastoid cells were cultured at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% fetal bovine sera (FBS), 2 mM glutamine, and 100 μg/ml penicillin-streptomycin. The media was replaced twice weekly and the cells proliferated in suspension. After 1 month, aliquots of the cells were frozen, and the remaining cells were subcultured weekly for further analysis.

The Mk adenovirus isolate was prepared directly from a crude cell lysate. Mk cells were washed twice in phosphate-buffered saline (PBS), resuspended at a concentration of 1×10^7 cells/ml in PBS, and subjected to three freeze-thaw cycles to lyse the cells. Debris was spun out and the supernatant harvested and stored at −70°C.

The Ad2 prototype was originally obtained from M. Horwitz (Albert Einstein College of Medicine, Bronx, NY). The A549 lung carcinoma cell line (CCL 185; American Type Tissue Collection, Rockville, MD) was used to propagate adenovirus.

Immunophenotyping

Analysis of cell surface antigens was carried out by two-color immunofluorescent flow cytometry using standard direct staining methods. Briefly, $1–3 \times 10^5$ cells were pelleted in each well of a 96-well V-bottomed plate (Dynatech, Chantilly, VA). Ten μl of the appropriate FITC-conjugated antibody was added to each well. After incubation at 4°C for 30 min, cells were washed and analyzed on a FACScan equipped with an argon ion laser and LYSIS II software support (Becton-Dickinson, San Jose, CA). The monoclonal antibodies (mAb) directed against HLA DR, CD3, CD19, CD20, and CD21 were purchased from Becton-Dickinson. Affinity purified goat antihuman IgG, IgM, IgA, IgD, lambda, and kappa (TAGO, Burlingame, CA) were also utilized. Nonspecific binding was monitored using isotypic controls.

EM Analysis

Mk cells (3×10^6) were washed in PBS, fixed in 2.5% glutaraldehyde-cacodylate, dehydrated in a graded series of methanol solutions, and embedded in epoxy resin as previously described [Harb, 1984]. Thin sections were stained with uranyl acetate followed by lead citrate and examined in a JEOL 100S transmission electron microscope.

Immunofluorescence Staining for Viral Antigens

Acetone-fixed cytospin slides were stained for adenovirus and EBV proteins by standard indirect immunofluorescence assay techniques. Mk was assayed for the presence of a productive EBV infection using a mAb vs. EBV envelope glycoproteins 350/300 and 250/200 (Accurate, Westbury, NY). The marmoset B95-8 cell line, with a productive EBV infection [Miller and Lipman, 1973], and the human Burkitt cell line Raji, which is EBV nuclear antigen (EBNA)-positive but does not produce virus [Epstein et al., 1966], were used as positive and negative controls, respectively. Expression of adenovirus proteins after infection of the EBNA-positive lymphoblastoid cell line HMy.C1R [Storkus et al., 1987] was assayed with the Bartels antiadenovirus mAb (Baxter, W. Sacramento, CA). Ad2-infected and uninfected A459 cells were used as positive and negative controls. Staining was detected with a Bartels FITC-conjugated goat antimouse IgG mAb (Baxter).

Adenovirus-Specific Immunohistochemical Assay

Adenovirus proteins were assayed on both formalin-fixed tissue sections and acetone-fixed cell spots with a rabbit anti-Ad2 penton sera (kindly provided by M. Horwitz). After preadsorption with PBS containing 2% goat sera, slides were incubated with the adenovirus-specific antibody (1:1,000 dilution) for 90 min at 37°C in a humidified chamber. Slides were washed in PBS three times and incubated with biotinylated goat anti-rabbit IgG (1:200 dilution) for 30 min. To detect staining, the Vectastain avidin-biotin complex method with an alkaline phosphatase detection system was employed according to the manufacturer's instructions (Vector Labs, Burlingame, CA). Each assay was run with either Ad2-infected and uninfected A549 cell spots or adenovirus-positive and negative tissue sections as positive and negative controls. Test slides were assayed with nonimmune rabbit sera as another negative control.

Viral Plaque Assay

Triplicate serial 10-fold dilutions of viral-infected cell lysates or intact Mk cells in DMEM (200 μl each) were adsorbed onto A549 monolayers in 60 mm dishes for 1 h at 37°C in a 5% CO_2 atmosphere. Monolayers were overlaid with 5 ml of DMEM containing 2% FBS, glutamine (2 mM), penicillin-streptomycin (100 μg/ml), fungizone (0.25 μg/ml), 2.5 mM $MgCl_2$, and 0.9% agarose. After incubation for 8 days, 3 ml of overlay containing .004% neutral red was added. Plates were then incubated in the dark and plaques counted on day 10.

Limiting Dilution Viral Culture Assay

Mk cells were washed five times and serial dilutions representing 100, 10, 1, and 0.1 cells in 100 μl DMEM were adsorbed for 1 h onto A549 monolayers in 24-well plates (8 wells each). As controls, two wells each were inoculated with 100%, 10%, and 1 50% tissue culture infectious doses ($TCID_{50}$) of the Ad2 prototype or media alone. Monolayers were incubated in DMEM, 2% FBS at 37°C in a 5% CO_2 atmosphere. Test wells were scored for the presence of CPE when the control wells with 1 $TCID_{50}$ of Ad2 developed 2+ CPE. The lowest dilution of Mk cells at which at least 50% of the wells were positive was used to calculate the proportion of Mk cells with a productive adenovirus infection.

CASE REPORT

The patient was a 4-year-old black male with pancytopenia and marrow evidence of myelofibrosis. Chromosomal analysis of bone marrow cells was normal. The

donor was the patient's brother, who was mismatched for one HLA-A, -B and -DQ antigen each, but was serologically matched for HLA-DR and compatible in a mixed lymphocyte culture assay. Both recipient and donor were positive for IgG antibody to EBV viral capsid antigen pretransplant by an indirect fluorescent assay performed at the Wisconsin State Laboratory of Hygiene. The conditioning regimen included total body irradiation (1,400 rads with lung shielding), busulfan, cytosine arabinoside, cyclophosphamide, and high-dose methylprednisolone. Graft vs. host disease prophylaxis consisted of in vitro T-cell depletion of the marrow inoculum with the mAb $T_{10}B_9$ and intravenous cyclosporine in the early posttransplant period [Ash et al., 1990]. The patient engrafted by day 33, but his course was complicated by acute graft vs. host disease, which was treated with methylprednisolone. Four months posttransplant, while still taking methylprednisolone and cyclosporine, he developed nasal congestion, pulmonary infiltrates, and fevers. Throat and stool cultures were positive for adenovirus. A cervical node biopsy was consistent with BLPD, and therefore, the cyclosporine was discontinued and the steroids were tapered. The patient subsequently developed upper airway obstruction due to lymphoproliferative disease involving the nasopharynx. The nasopharyngeal mass was partially resected on day 148 (also adenovirus culture-positive), and treatment with vincristine (1.5 mg/M^2) and cyclophosphamide (400 mg/M^2) was initiated. Unfortunately, the tumor extended to occlude the left cavernous sinus, the pulmonary infiltrates progressed, and patient expired on day 165 posttransplant. At autopsy, there was extensive involvement of the lymph nodes and multiple organs, including the lung, gastrointestinal tract, and adrenals, with a diffuse lymphoproliferative process. Cell surface marker analysis of the lymphoma cells was compatible with a B-cell origin. In addition, the lung was culture-positive for adenovirus and contained scattered alveolar cells with nuclear inclusion bodies consistent with adenovirus pneumonia.

RESULTS

Focal Areas of the Lymphoma Stain Positive for Adenovirus Proteins

Tissue sections obtained from the patient's autopsy were examined with an adenovirus-specific immunohistochemical stain. In the lung, scattered alveolar cells and bronchioles were positive for adenovirus proteins. In addition, an area of the lymphoma infiltrating the lung stained strongly positive for adenovirus. Figure 1 shows a representative field of the adenovirus-positive lymphoma cells in the lung tissue. In contrast, the lymphoma infiltrating the stomach and duodenum was negative for adenovirus proteins (data not shown).

Mk Is an Adenovirus-Positive EBV-Transformed B-Cell Line

The Mk cell line established from the patient's pleural fluid was confirmed to have features consistent with an EBV-associated malignant B-cell lymphoma. FACS analysis documented the presence of HLA DR, lambda light chains, and the B-cell surface antigens CD19, CD20, and CD21. The Mk cell line was positive for EBNA (undertaken by W. Henle Children's Hospital of Philadelphia, PA) and negative for EBV envelope glycoproteins by immunofluorescent staining, characteristic of a latent EBV infection. Chromosome analysis revealed multiple numeric and structural anomalies, including trisomy 9 and abnormalities of chromosomes 8, 12, and 22, consistent with a clonal neoplastic process (carried out by the Mayo Clinic, Rochester, MN).

Histocompatability typing demonstrated the Mk cell line to be of donor origin. The Mk cell line was originally found to produce adenovirus by routine viral culture on HEp-2 cells in the clinical laboratory. The cell line was passaged for up to 6 months without significant cell lysis (< 5–10%).

Mk Contains Adenovirus Virions in Both Nucleus and Cytoplasm

Several unusual structural features of the adenovirus infection in the Mk cell line were identified by EM. In a lytic infection of a permissive cell, virions assemble and remain in the nucleus until the cell lyses [Horwitz, 1990]. In contrast, as illustrated in Figure 2, adenovirus virions were found in both the nucleus and the cytoplasm of some of the Mk cells. Intracytoplasmic virions were found in multiple, otherwise healthy looking cells and thus are unlikely to represent an artifact. In other cells, virions were primarily localized in the nucleus. Another atypical finding was the presence of nonvirion paracrystalline inclusions in both the nucleus and cytoplasm of some of the cells (inset, Fig. 2). Similar structures have been described in the nucleus of permissive cells infected in vitro with certain adenovirus isolates, but their composition was not identified [Weber and Stich, 1969]. Consistent with latent EBV infection, EBV particles were not visualized in the Mk cell line by EM.

Mk Adenovirus Isolate Is Cytopathic in Permissive Cells

The Mk adenovirus isolate was tested for the ability to infect other cell lines in order to evaluate its phenotype. The adenovirus produced by Mk readily killed the permissive A549 lung epithelial cell line i.e. typical adenoviral CPE developed over a 4-day period after inoculation of a A549 monolayer in a T25 flask with 100 μl of the Mk cell lysate. These data demonstrate that the adenovirus isolate is not a mutant with reduced pathogenicity. In addition, the Mk isolate was not more lymphotropic than the Ad2 prototype. Both Mk and Ad2 viral isolates infected $< 5\%$ of cells in the lymphoblastoid cell line HMy.C1R as assayed by immunofluorescent staining.

Mk Produces High Level of Infectious Adenovirus

The titer of the adenovirus produced by Mk was determined by a viral plaque assay on A549 cells. A Mk crude cell lysate was directly titered and compared with a crude

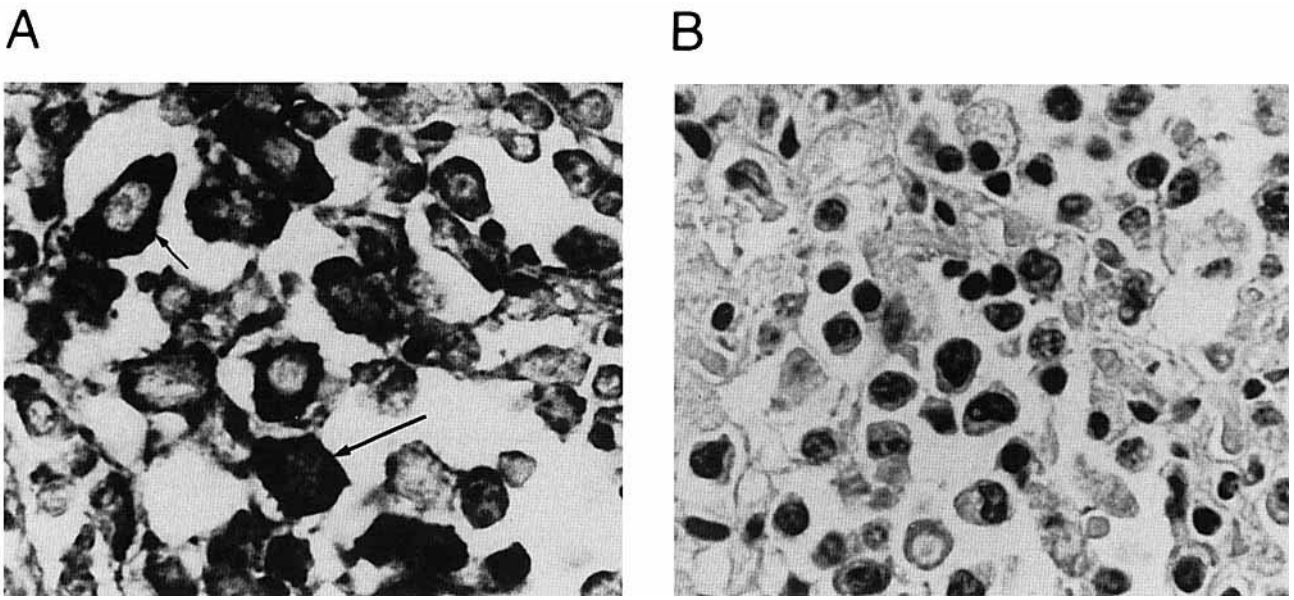

Fig. 1. Lymphoma cells infiltrating the lung are infected with adenovirus. **A:** Adenovirus-specific immunohistochemical stain. The majority of cells in this field stain positive for adenovirus proteins. Staining is primarily cytoplasmic (short arrow) in some cells. Other cells exhibit both nuclear and cytoplasmic staining (long arrow). Magnification = 400×. **B:** Hematoxylin and eosin stain of adjacent tissue section. The lung parenchyma is obscured by a mixed population of lymphoid tumor cells with plasmacytoid and follicular center cell morphology. Magnification = 400×.

lysate of Ad2-infected A549 cells. Mk was found to chronically produce ~ 1×10^3 plaque-forming units (pfu) of adenovirus per cell, which was similar to the yield from a 24-hour lytic adenovirus infection in A549 cells (2×10^3 pfu/cell).

Majority of Mk Cells Express Adenovirus Proteins and Produce Infectious Virions

Mk cells were stained for adenovirus antigens and a limiting dilution viral culture was performed in order to determine the proportion of cells expressing adenovirus proteins and producing infectious virus. Approximately 90% of Mk cells were positive for adenovirus proteins by the adenovirus-specific immunohistochemical assay used to stain tissue sections described above (data not shown). In addition, a limiting dilution analysis of intact Mk cells cultured on A549 monolayers revealed that virtually 100% of Mk cells produced infectious adenovirus. These data indicate that the Mk cell line maintains a uniform, productive adenovirus infection.

DISCUSSION

A spontaneous, adenovirus-positive B-cell line established from an immunocompromised patient with both EBV-associated BLPD and adenovirus pneumonia was characterized. This child received a T-cell-depleted bone marrow transplant from a related, partially mismatched donor, which are both risk factors associated with the development of BLPD [Shapiro et al., 1988]. The fact that both the recipient and donor were positive for EBV-specific antibody pre-transplant suggests that the lymphoproliferative disease developed as a result of reactivation of latent EBV infection. At autopsy, there was evidence of adenovirus pneumonia and a malignant B-cell lymphoma, as well as direct infection of the lymphoma by adenovirus. A focus of the lymphoma infiltrating the lung was strongly positive for adenovirus proteins by immunohistochemical staining, whereas the lymphoma infiltrating the adjacent lung tissue, stomach, and duodenum was negative for adenovirus antigens. Therefore, it appears that the adenovirus infected the lymphoma after the evolution of the EBV-driven lymphoproliferation into an oligoclonal or monoclonal neoplastic process in this patient.

This is the first study to document a de novo, persistent adenovirus infection in a human cell type. The Mk lymphoblastoid cell line was found to exhibit a uniform, highly productive adenovirus infection. Moreover, the adenovirus-infected cell line was maintained in long-term culture without cell death. Previous studies have found that experimental adenovirus infection of most B- and T-cell lines is inefficient, and only low level persistence (involving < 5% of cells) has been documented [Silver and Anderson, 1988]. Therefore, the Mk cell line provides a unique opportunity to study the mechanisms of persistent adenovirus infection.

The identification of a persistent, productive adenovirus infection in this cell line proves that adenovirus replication is not sufficient for cell lysis. No difference was found in the cytopathogenicity of the Mk adenovirus isolate compared to the Ad2 prototype to account for the absence of cell lysis in Mk cells. Zhang and colleagues [1994] have shown that the inhibition of cellular protein synthesis during the late phase of adenovirus infection is a crucial step for cell lysis and is associated with dephosphorylation of the cellular translation initiation factor eIF-4E. Certain cell lines that are resistant to host protein synthesis shut off are not lysed by adenovirus

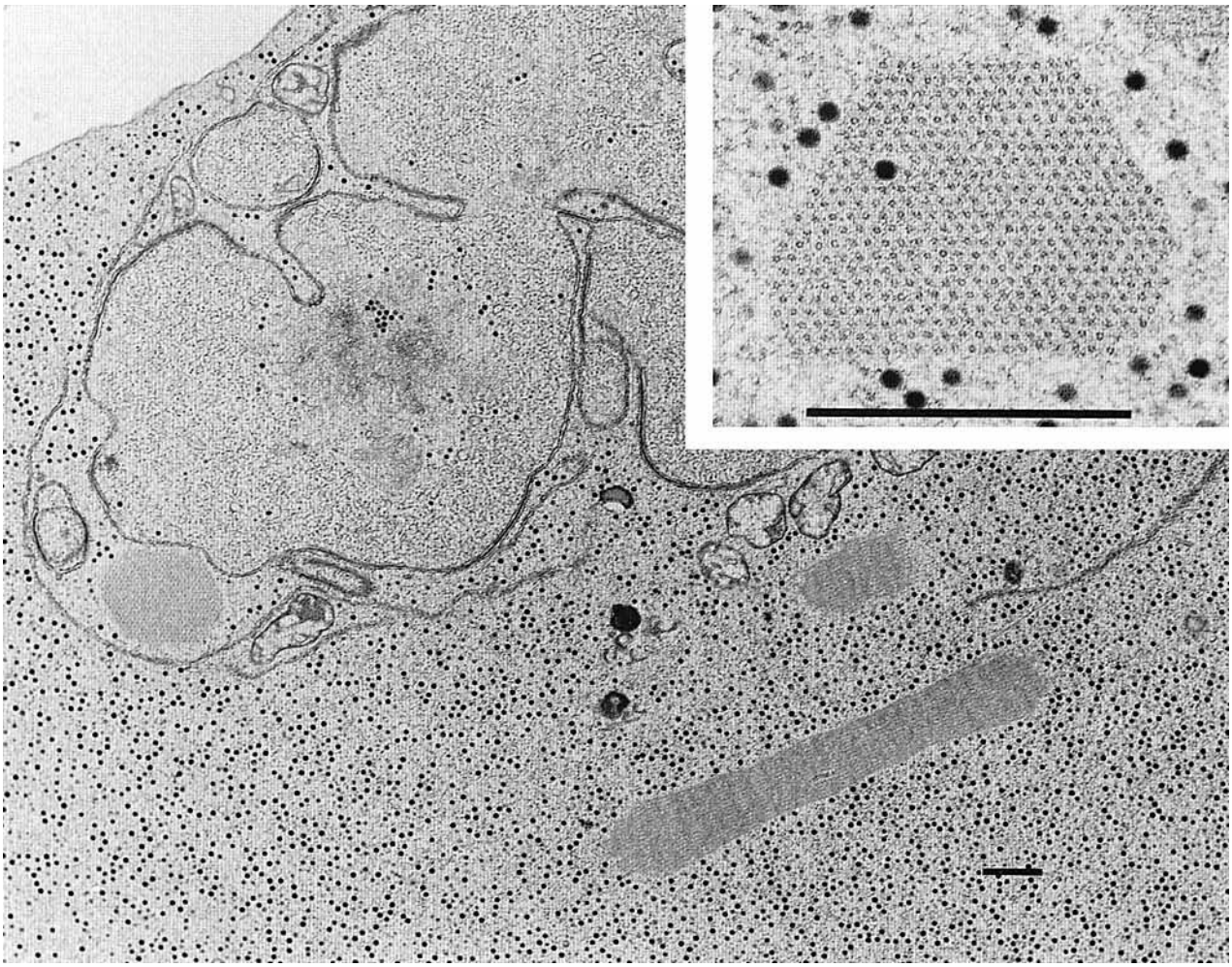

Fig. 2. Electron micrograph of a Mk cell. Adenovirus virions are present in the lobated nucleus and fill the cytoplasm. Three paracrystalline inclusions are seen in the cytoplasm. Magnification = 9,000×; bar = 1 μm. **Inset:** Adenovirus virions surround an intranuclear paracrystalline inclusion. The tubular subunits are oriented in cross section and are arranged hexagonally. Magnification = 46,000×; bar = 1 μm.

[O'Malley et al., 1989]. Therefore, one postulate is that Mk may be resistant to adenovirus-induced inhibition of cellular protein synthesis, perhaps related to either overexpression of eIF-4E or resistance of eIF-4E to adenovirus-induced dephosphorylation. This hypothesis is consistent with the atypical appearance of the adenovirus infection in the Mk cell line on EM. The presence of intracytoplasmic virions may reflect continuous viral production without inhibition of cellular protein synthesis, allowing virions to exit the nucleus after assembly. Similarly, the paracrystalline aggregates may represent excess viral structural proteins which accumulate over time.

In addition, these data support a role for persistent adenovirus infection in vivo. In the case described in this report, in which adenovirus infection was first documented 4 months posttransplant, it was not possible to document whether this was a primary infection or reactivation of latent infection. However, persistent adenovirus infection may be an important source of adenovirus disease in immunocompromised hosts. The specific role of primary B-cells and/or EBV-transformed B-cells as reservoirs of adenovirus infection remains to be determined. Although it is conceivable that EBV transformation could make B-cells more susceptible to adenovirus infection, no difference was found in the efficiency of experimental adenovirus infection in a panel of EBV-positive and EBV-negative B-cell lines by Lavery et al. [1987]. The role of other human cell types in which adenovirus may establish persistent infections in vivo and the mechanisms involved need to be evaluated. Furthermore, this information will be relevant to the use of adenovirus as a vector for gene therapy and recombinant vaccines.

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